IDENTIFICATION OF PROTEINS LOCATED IN THE NEIGHBOURHOOD OF THE BINDING SITE FOR THE ELONGATION FACTOR EF-Tu ON *ESCHERICHIA COLI* RIBOSOMES

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1. Introduction

The involvement of EF-Tu in protein synthesis has been demonstrated by a number of experiments [1–5], and by the use of labelled EF-Tu [6] it can be shown directly that the factor becomes actually bound to the ribosomal 50 S subunits during the elongation process.

The present report describes the identification of 50 S proteins which are in the neighbourhood of the binding site for EF-Tu. The identities of these proteins were determined by cross-linking EF-Tu to radioactive 50 S subunits with the bifunctional reagent methyl-4-mercapto-butyrimidate. After cross-linking, the proteins were extracted and applied to a Sepharose column to which antibodies against EF-Tu were bound. Identification of the proteins cross-linked to EF-Tu was made by two-dimensional gel electrophoresis, followed by measurement of radioactivity. In this way proteins L23, L28 (L1, L3 and L24) were found to be present in the cross-linked complex between EF-Tu and ribosomal proteins, and it can be concluded that these proteins are located in the neighbourhood of the ribosomal binding site for EF-Tu.

2. Materials and methods

2.1. *Materials*

E. coli strain CGSC 2834/a was grown in 1% tryptone, 0.5% yeast extract and 0.2% glucose at 37°C. Ribosomal subunits, EF-Tu [7] and [^{14}C]Phe-tRNA [8] (specific activity: 0.4–0.8 μmol [^{14}C]Phe/μmol tRNA) were prepared as described. 50 S subunits were labelled with [^{3}H]borohydride to a specific activity of 54 Ci/mmol [9] and EF-Tu to a specific activity of 5–10 Ci/mmol [6,10]. Assays for [^{3}H]GDP binding of EF-Tu [11], binding of [^{14}C]Phe-tRNA and [^{3}H]EF-Tu to ribosome [6] and polyphenylalanine synthesis [12] as well as isolation of the 70 S–EF-Tu complex [6] were carried out as described. [^{3}H]Borohydride (6–40 Ci/mmol) was purchased from Amersham-Buchler, Braunschweig. [^{3}H]GDP (1.29 Ci/mmol) and [^{14}C]Phe-tRNA (460 mCi/mmol) were obtained from NEN Chemicals, Dreieichenhain), methyl-4-mercapto-butyrimidate from Pierce Eurochemie, Rotterdam, and β,γ-methylene-guanosine-5′-triphosphate (GMP-PCP) from Miles Laboratories.

2.2. *Methods*

Electrophoretically pure EF-Tu was injected into rabbits by Dr C. Sorg. In immuno-electrophoresis the antibodies migrated more towards the anode at pH 8.5 than did human serum albumin and they precipitated as a single band with EF-Tu. In the radio-immune-assay antibodies against EF-Tu precipitated with [^{3}H] EF-Tu while non-immune serum did not.

2.3. *Cross-linking of EF-Tu and 50 S subunits*

EF-Tu or [^{3}H]EF-Tu or 50 S subunits were treated with methyl-4-mercapto-butyrimidate (MBI). The cross-linking reaction with this bifunctional reagent was carried out in two steps [13]: viz., binding of MBI to free amino groups of proteins, and oxidation of the bound MBI molecules to form the disulfide bridges. For EF-Tu or [^{3}H]EF-Tu the reaction was carried out for 20 min at 0°C in 1 ml with 365 μg EF-Tu or [^{3}H]EF-Tu and 5.5 mg bovine serum albumin, 50 mM triethanolamine–HCl (TEA-HCl), pH 8.0, 5 mM MgAc, 50 mM KCl, 3% β-mercapto-

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ethanol, 10 mM MBI. To remove excess MBI the reaction mixture was dialysed against 2 X 2 liter of 50 mM TEA–HCl, pH 8.0, 5 mM MgAc, 50 mM KCl, 3% β-mercaptoethanol. The reaction product was denoted 'treated reduced' EF-Tu or [^{3}H]EF-Tu. The molecular weights of the cross-linked oxidised and cross-linked reduced proteins were determined on a Biogel P-150 column (0.8 X 30 cm). The elution buffer contained 50 mM Tris–HCl, pH 8.2, 0.5 M LiCl, 1% bovine serum albumin. The elution rate was 3 ml/h. Fractions of 150 μl were collected and the radioactivity was measured on a portion of each fraction.

For the 50 S subunits the cross-linking reaction was carried out as described for EF-Tu, but with 2 mM instead of 10 mM MBI. The sedimentation behaviour of treated oxidised and treated reduced 50 S subunits was determined on a linear 12–30% sucrose gradient. The samples were centrifuged at 4°C in a SW 40 rotor for 15 h at 23 000 rev/min. Fractions of 350 μl were collected and the absorbance at 260 nm was determined. Both treated reduced 50 S subunits and treated reduced EF-Tu (or [^{3}H]EF-Tu) were incubated together with 30 S subunits, polyU, GMP-PCP and [^{14}C]Phe-tRNA to form the treated 70 S–EF-Tu complex. Details are given in the legend to fig.1. After sedimentation through 5% sucrose this complex was treated for 20 min at 0°C with 40 mM H_2O_2 to oxidise the SH-groups.

The treated oxidised (i.e. cross-linked) 70 S–EF-Tu complex was washed for 20 min at 0°C with 1 M NH_4Cl and centrifuged through a linear 12–30% sucrose gradient at 4°C in a SW 40 rotor for 15 h at 23 000 rev/min. The ribosomal proteins were extracted from the isolated 70 S–EF-Tu complex by acetic acid [14]. The proteins cross-linked to EF-Tu were separated from the other ribosomal proteins by affinity chromatography through a Sepharose 4B column (0.8 X 8 cm) [15] loaded with antibodies against EF-Tu [16].

300 μg of radioactive proteins extracted from the [^{3}H]70 S–EF-Tu complex were applied onto the column in 20 mM Tris–HCl pH 7.8, 1 M LiCl and 1.2 M urea, and the loaded column was allowed to stand for 2 h at 4°C. Unbound material was eluted with the buffer containing Tris, LiCl and urea (see above) and bound material with 8 M urea, pH 2.0. The cross-links were cleaved by reduction with 3% β-mercaptoethanol and the single ribosomal proteins produced were identified by two-dimensional gel electrophoresis [17]. For each plate 600 μg unlabelled 50 S proteins were used as 'carrier' proteins. All protein spots were cut out, solubilised in 1 ml soluene for 5 h at 40°C, and the radioactivity was determined.

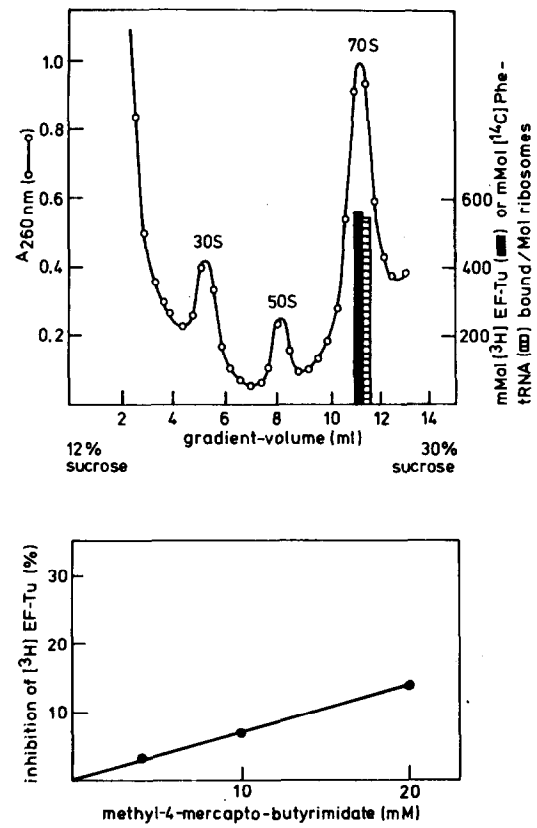

Fig.1. Inhibition of biological activity of EF-Tu and [^{3}H]EF-Tu as a function of MBI concentration. 165 pmol 50 S, 186 pmol 30 S, 12.5 μg polyU, 20 mM Tris–HCl pH 7.8, 10 mM MgAc, 4 mM dithiothreitol were preincubated in 250 μl for 10 min at 30°C. After cooling 1.36 μg EF-Tu, 50 mM NH_4Cl, 40 μM GMP-PCP and 150 pmol [^{14}C]Phe-tRNA were added and the solution brought to a volume of 500 μl with water. The incubation was carried out for 10 min at 30°C. The 70 S–EF-Tu$_{SH}$ complex was applied to a 12–30% sucrose gradient in 20 mM Tris–HCl pH 7.8, 20 mM MgAc, 0.2 mM GMP-PCP and centrifuged at 4°C in an SW 40 rotor for 15 h at 23 000 rev/min. After fractionation of the gradient the absorption at 260 nm and the radioactivity of each fraction were determined.

3. Results and discussion

3.1. *Control experiments*

(1) EF-Tu or [^{3}H]EF-Tu was treated with different concentrations of MBI. The treated EF-Tu was kept under reducing conditions and its activity was tested by [^{14}C]Phe-tRNA binding to 70 S ribosomes. After treatment with 10 mM MBI, the biological function of EF-Tu was preserved: the inhibition was less than 10% (fig.1).

(2) Treated EF-Tu or [^{3}H]EF-Tu was oxidised and its molecular weight determined on a Biogel P-150 column. The total radioactivity from treated oxidised and treated reduced [^{3}H]EF-Tu was eluted in the same position as that of untreated [^{3}H]EF-Tu. This shows that during the cross-linking process no dimers or greater aggregates of [^{3}H]EF-Tu had been formed.

(3) 50 S subunits were cross-linked in the same way as EF-Tu. After treatment with 10 mM MBI, the 50 S subunits lost their ability to bind [^{14}C]Phe-tRNA. Therefore, in further experiments 50 S subunits treated with 2 mM MBI were used to form the 70 S–EF-Tu complex in the presence of untreated 30 S, treated reduced EF-Tu, GMP-PCP, and [^{14}C]Phe-tRNA. The inhibition in this case was only 20–30%.

(4) The sedimentation behaviour of 50 S subunits treated with 2 mM MBI, both in the oxidised and treated form, was compared with that of untreated 50 S subunits by centrifugation through a linear sucrose gradient. All the samples showed a normal sedimentation profile which reveals that the cross-linking reaction did not induce either aggregation or a detectable conformational change in the 50 S subunits.

(5) The cross-linked 70 S–[^{3}H]EF-Tu complex was dissociated at low Mg^{2+}-concentration and the resulting subunits were separated by sucrose gradient centrifugation. The profile obtained was identical to that of untreated 30 S and 50 S subunits, showing that the subunits were not cross-linked to one another and that there was no detectable change in conformation.

(6) It was necessary to show that [^{3}H]EF-Tu becomes covalently bound to MBI-treated ribosomes after oxidation of the 70 S [^{3}H]EF-Tu complex. Gordon et al. [18] have shown that EF-Tu dissociates from ribosomes after treatment with 1 M NH_4Cl. If EF-Tu is bound covalently to the ribosome, this dissociation should be inhibited. In order to test this, the 70 S–[^{3}H]EF-Tu complex was formed with treated reduced 50 S subunits, untreated 30 S subunits, polyU, treated reduced [^{3}H]EF-Tu, GMP-PCP, and [^{14}C]Phe-tRNA. The complex was isolated by centrifugation through 5% sucrose and oxidised. The oxidised 70 S–[^{3}H]EF-Tu complex was adjusted to 1 M NH_4Cl, kept for 20 min at 0°C, and then centrifuged through a linear gradient of 12–30% sucrose. In the case of the cross-linked oxidised 70 S–[^{3}H]EF-Tu complex, 89% EF-Tu remained bound to the ribosomes after treatment with NH_4Cl, while in the case of untreated 70 S–[^{3}H]EF-Tu complex no [^{3}H]EF-Tu could be found on the ribosomes after washing. Reduction of the cross-linked oxidised 70 S–[^{3}H]EF-Tu complex with β-mercaptoethanol resulted in the complete dissociation of the elongation factor from the ribosomes after treatment with NH_4Cl. From these experiments it was concluded that EF-Tu could be successfully cross-linked to ribosomal proteins.

(7) As expected, the binding of untreated EF-Tu to 70 S ribosomes depended on the presence of [^{14}C]Phe-tRNA and GMP-PCP. In the absence of 30 S subunits or of polyU or when heat-inactivated 50 S subunits were used, neither EF-Tu nor [^{14}C]Phe-tRNA was bound. Using treated 50 S subunits and treated [^{3}H]EF-Tu, both in the reduced form, the same amount of EF-Tu and Phe-tRNA binding was observed as when untreated 50 S or EF-Tu were used. In all cases 540–580 mmol [^{3}H]EF-Tu and [^{14}C]Phe-tRNA were bound per mole of ribosomes. The results show that the specificity of EF-Tu binding to ribosomes was not changed by treatment of 50 S subunits or [^{3}H] EF-Tu with methyl-4-mercapto-butyrimidate.

3.2. *Identification of cross-linked proteins*

The 50 S proteins cross-linked with EF-Tu were separated from the other ribosomal proteins by an affinity chromatography step [15] which was carried out using a column of Sepharose 4B to which antibodies against EF-Tu were bound [16]. After formation of the cross-linked 70 S–EF-Tu complex and extraction of the proteins (see Methods) one half of the protein mixture was separated on a column loaded with antibodies against EF-Tu and the other half on a column loaded with non-immune serum. The eluted proteins from each column were subjected to reductive cleavage and analysed by two-dimensional gel electrophoresis. The protein spots were cut out from the gel,

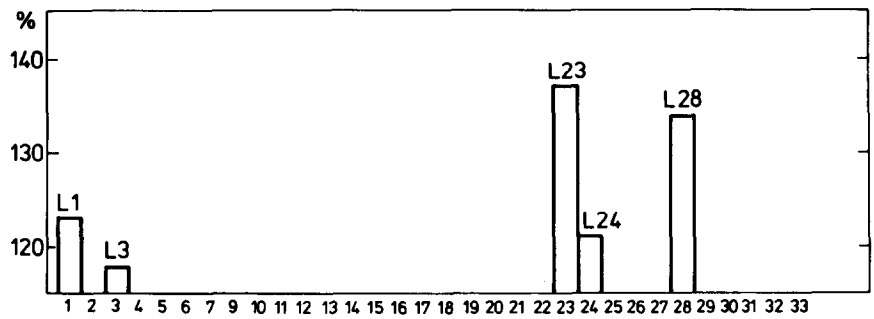

Fig.2. Ribosomal proteins in the cross-linked EF-Tu 50 S protein complex. *Abscissa*: 50 S ribosomal proteins (L1, L2 etc.). *Ordinate*: amount of radioactive protein recovered from the two-dimensional gel onto which the sample from the anti-EF-Tu loaded Sepharose column was applied, as compared to the amount of protein from the gel with the sample from the 'non-immune serum' column.

solubilised in soluene and their radioactivity was determined. The radioactivity of each 50 S protein from the non-immune serum column was denoted as 100%, and the radioactivities of all the proteins eluted from the corresponding anti-EF-Tu-loaded Sepharose-column were related to these values.

The results of eight such experiments were combined and the mean values obtained for each protein are shown in fig.2. It can be seen that for proteins L1, L3, L23, L24 and L28 the mean values lie outside the range of experimental variation, which was estimated as ± 15%. Statistical calculation showed that the significance values for proteins L23 and L28 were higher than those for proteins L1, L3 and L24. Therefore, it can be concluded with higher confidence for the first than for the second group of ribosomal proteins that they are cross-linked to EF-Tu.

The relatively long distance spanned by the bifunctional reagent might explain why protein L7/L12 are not found among the cross-linked proteins (fig.2), although they are involved in the binding of EF-Tu (see ref. [19] for review). Indeed, by using the shorter bifunctional reagent *p*-nitrophenyl-chloroformate, L7/L12 have recently been found among several other proteins to be cross-linked to EF-Tu [20]. Therefore, it seems reasonable that in the present study with the long bifunctional reagent, proteins were identified which are in the neighbourhood of the binding site for EF-Tu on the 50 S subunit, but are not necessarily directly in contact with this factor when it is bound to the ribosome. Further, the possibility cannot be excluded that one or more of the proteins identified (fig.2) are cross-linked indirectly, i.e. via another protein, to EF-Tu.

It is interesting to compare the data described in this paper with the topographical 50 S model [21,22] derived from immune-electronmicroscopy. In this model the proteins L1 and L23 are at least partly located in a central region (the 'seat') of the particle. The same ribosomal region has been concluded to be involved in the binding of EF-Tu by the work of San José et al. [20]. It remains to be seen whether the proteins L3, L24 and L28, whose locations on the ribosome have not yet been determined by immune-electronmicroscopy, are also located in the 'seat' region of the 50 S particle.

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